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Activation of pig and cattle oocytes by butyrolactone I: morphological and biochemical study

Michal Kubelka[1], *Martin Anger*[1], *Antonín Pavlok*[1], *Jaroslav Kalous*[1], *Richard M. Schultz*[2] *and Jan Motlík*[1]

Institute of Animal Physiology and Genetics, Liběchov, Czech Republic, and University of Pennsylvania, USA

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Summary

In this study a specific inhibitor of cyclin-dependent kinases (cdks), butyrolactone I (BL I), was used for activation of pig and cattle metaphase II (MII) oocytes. BL I at a concentration of 100 µM was able to induce activation of both pig and cattle MII oocytes in a manner dependent on exposure time; however, precise timing of BL I exposure was required for the best activation results. The optimum activation rates were obtained when cattle MII oocytes were treated for 5 h with BL I and subsequently for 3–11 h in control medium, and pig MII oocytes for 8 h in BL I and then for 8–16 h in control medium; the percentage of activated oocytes after such treatment varied between 55% and 74% and between 53% and 81% for cattle and pig oocytes, respectively. Shorter exposures to BL I led to re-entry of the oocytes to the metaphase state in 35–50% of oocytes, the remaining oocytes forming a pronuclear stage; longer exposure to BL I led to increased numbers of oocytes being abnormal or degenerated. The behaviour of histone H1 kinase and mitogen activated protein (MAP) kinase, also measured during the experiment, reflected the morphological changes in the oocytes: both were inactivated after BL I treatment, though the inactivation of histone H1 kinase occurred 2 h ahead of that of MAP kinase. However, in the oocytes treated for a shorter time with BL I, with the reoccurrence of condensed chromatin in proportion of the oocytes cultured in control medium after BL I treatment, both kinases became reactivated. Taken together, these results suggest the possibility of using BL I for activation and cloning experiments in both species.

Keywords: Activation, Butyrolactone I, MII oocytes, MAP and H1 kinase

Introduction

After the completion of maturation, mammalian oocytes remain arrested at the metaphase II (MII) stage until fertilisation or artificial activation. This rather long meiotic block is caused by the presence of high levels of M-phase promoting factor (MPF) activity. MPF has been shown previously to be a complex of a catalytic subunit, cdc2 kinase and its regulatory subunit cyclin B, and its activity can be measured as histone H1 kinase activity (for review see Nurse, 1990; Pines & Hunter, 1990). High MPF activity in MII oocytes is in turn maintained by the so-called cytostatic factor (CSF). Several different proteins have been shown in vertebrate oocytes to possess CSF activity; in mammals the presence of two of these proteins has been detected in MII oocytes: the proto-oncogene product c-mos and mitogen activated protein (MAP) kinase (Haccard *et al*., 1993; Verlhac *et al*., 1996; Ciemerych *et al*., 1999). The mechanism by which CSF maintains high levels of cdc2 kinase activity is not clear; however, it is believed that CSF most probably inhibits the regulatory subunit (cyclin B) degradation machinery and slows down cyclin B destruction. The results of experiments performed in *Xenopus* (Abrieu *et al*., 1996, 1997; Thibier *et al*., 1997) and mouse (Verlhac *et al*, 1996) suggest that the CSF-mediated inhibition of cyclin destruction is exerted via MAP kinase activation by c-mos (which itself is a protein kinase). The degraded cyclin can be replaced by continuously synthesised new protein and it can participate in the maintenance of MPF activity.

All correspondence to: Michal Kubelka, Institute of Animal Physiology and Genetics, Academy of Sciences of the Czech Republic, CZ-277 21, Liběchov, Czech Republic.

[1]Institute of Animal Physiology and Genetics, 277 21 Liběchov, Czech Republic.

[2]Department of Biology, University of Pennsylvania, Philadelphia, PA 19104–6018, USA.

Under physiological conditions, when MII oocytes are fertilised the spermatazoon firstly fuses with egg plasma membrane and then the activation of the matured egg occurs (including release from MII block, extrusion of the second polar body and pronuclear formation). The sperm triggers a series of Ca^{2+} oscillations, which have been proven to be an essential activating signal (Homa *et al*., 1993). Ca^{2+} influences the cyclin degradation machinery and it has been shown that it acts through activation of Ca^{2+}-calmodulin dependent kinase II (CaMKII), which in turn activates the ubiquitin-dependent cyclin degradation pathway (Lorca *et al*., 1993; Abrieu *et al*., 1996).

Apart from electrical stimulation, a number of different chemical substances have been used for artificial activation of mammalian oocytes. The basic idea in using these different substances is either to increase the intracellular calcium levels in order to mimic the Ca^{2+} oscillations accompanying fertilisation, or to influence the levels and/or the activities of proteins related to cell cycle regulation. However, while quite high rates of activation of mouse MII oocytes could be achieved by using representatives of both the above-mentioned groups, including ethanol (Cuthberson, 1983), Ca^{2+} ionophores or chelators (Steinhardt *et al*., 1974; Kline & Kline, 1992), SrC12 (Kono *et al*., 1996; Tateno & Kamiguchi, 1997) and the protein synthesis inhibitors cycloheximide and puromycin (Siracusa *et al*., 1978; Clarke & Masui, 1983), activation of porcine and, especially, cattle MII oocytes tends to be more complicated. Aged MII oocytes of these species respond similarly to mouse oocytes to these stimuli and can easily be activated by ethanol (Nagai *et al*., 1987), electrical stimulation or Ca^{2+} ionophore (Ware *et al*., 1989; Hagen *et al*., 1991), and in some cases by inhibition of protein synthesis (Fulka *et al*., 1991); however, younger oocytes are not activated by such treatment or their developmental potency is impaired. Such an influence of age on oocyte activation has been described also in mouse (Kubiak, 1989), but it is more pronounced in pig (Hagen *et al*., 1991), and especially in cattle oocytes (Ware *et al*., 1989), probably due to the longer time of oocyte maturation in these species. Fertilisation of aged bovine oocytes also resulted in asynchrony between female and male chromatin activation (decondensation) and pronuclear formation (Pavlok *et al*., 1997).

Much better results in activating pig and cattle oocytes have been achieved more recently by using the combination of Ca^{2+} elevating agents with either various inhibitors of protein kinases or inhibitors of protein synthesis. The number of various combinations include activation of cattle oocytes by treatment with ionomycin/ionophore together with 6-dimethylaminopurine (6-DMAP) (Susko-Parrish *et al*., 1949; Liu *et al*., 1998), activation of porcine oocytes by a combination of ethanol and 6-DMAP (Leal & Liu, 1998), or the simultaneous use of ionomycin/ionophore/ethanol + cycloheximide for activation of porcine (Cha *et al*., 1997) or cattle oocytes (Presicce & Yang, 1994; Tanaka & Kanagawa, 1997; Ernst *et al*., 1999). While the treatment of oocytes with ionomycin/ionophore or ethanol influences the intracellular Ca^{2+} levels and in such a way subsequently affects the CSF degradation pathway, 6-DMAP and cycloheximide/puromycin are likely to influence the activity and levels of cell-cycle-related protein kinases. The abrupt decrease in histone H1 kinase after oocyte activation has been reported in number of mammalian species including mouse (Verlhac *et al*., 1994; Moos *et al*., 1995, 1996), rabbit (Collas *et al*., 1995), cattle (Collas *et al*., 1993; Liu *et al*., 1998) and pig (Kikuchi *et al*., 1995). On the other hand the activity of one of the kinases involved in CSF, the MAP kinase, has been reported to remain elevated for several hours after an activation stimulus (Liu *et al*., 1998; Moos *et al*., 1996) and its inactivation could rather be correlated with pronuclear formation, at least in mouse zygotes (Moos *et al*., 1996).

In this study we report the use of a specific inhibitor of cdk kinases, butyrolactone (BL I), for activation of pig and cattle oocytes and we describe the changes in the activities of two cell-cycle-related kinases – cdc2 and MAP kinase – during activation.

Materials and methods

Materials

The following chemicals were used: BL I was from Funakoshi (Tokyo, Japan), histone H1 from Sigma (St Louis, MO), crystallised cattle albumin, silicone oil, sodium pyruvate, calcium lactate and Coomassie Blue from Serva (Heidelberg, Germany), TCM 199 (10× concentrated stock) and $NaHCO_3$ (7.5% stock) from Sevac (Prague, Czech Republic), fetal calf serum (FCS) from Bioveta (Ivanovice, Czech Republic), porcine follicle stimulating hormone (radio-iodination grade) (FSH) from Biogenesis (Poole, UK), Suigonan PG-600 (FSH/LH gonadotropins) from Intervet, International (Boxmeer, Holland), [γ-^{32}P]ATP from Amersham (London, UK), myelin basic protein (MBP), and polyvinyl alcohol (mol. wt. 300 000–50 000) and all other reagents from Sigma (St Louis, MO). Deionised and Nanopure filtered water was used for all media. The plastic dishes were from Nunclon (Roskilde, Denmark).

Oocyte collection and culture

Cattle ovaries, collected from slaughtered cows, were transported in physiological saline at 20 °C to the laboratory. The ovaries were then briefly washed for 20 s in 70% ethanol and twice in physiological saline. Follicles

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2.5–8 mm diameter were dissected with fine scissors, placed in 90 mm Petri dishes with the basic culture medium (see below), punctured and pressed with a bent preparation needle to isolate the oocytes. For oocyte culture *in vitro*, only healthy-looking oocyte–cumulus cell complexes (OCCs) were selected (Pavlok *et al.*, 1992). The OCCs were washed twice in the basic culture medium and transferred into 4-well Nunclon dishes with 0.5 ml medium under silicone oil. OCCs were cultured in 9.4 ml TCM 199 (10× concentrated stock), 2.1 ml $NaHCO_3$ (7.5% stock) in 100 ml H_2O and supplemented with 9.5 mM HEPES, 1.82 mM sodium pyruvate, 50 IU/ml penicillin potassium salt, 50 IU/ml streptomycin sulfate, 125 ng/ml amphotericin B (basic culture medium). This basic culture medium was supplemented with 3 mg/ml crystallised bovine serum albumin (BSA) and 10 IU FSH/LH gonadotropins (PG-600). All cultures of cattle oocytes were done in a humidified atmosphere composed of 5% CO_2, 10% O_2 and 85% N_2 at 39 °C.

Pig oocytes were obtained from ovaries of slaughtered non-cycling gilts of large breeds by aspiration of antral follicles about 5 mm in diameter. Only oocytes surrounded by compact cumuli were used for the culture. Oocytes were cultured in 0.1 ml droplets of basic culture medium, supplemented with 10% FCS and 0.5 IU/ml FSH, under paraffin oil at 38 °C, under 5% CO_2.

In the experiments examining the effect of BL I, the basic culture medium containing either BSA and PG-600 (for cattle oocytes) or FCS and FSH (for pig oocytes) was supplemented with 100 µM BL I, the dose determined by us previously (Kubelka *et al.*, 2000) as the most effective and still highly specific for cdk kinases; BL I was prepared as 50 mM stock solution in dimethylsulfoxide (DMSO).

Fixation, staining of oocytes and morphological analysis

For checking the current stage of maturation and activation at the time of sample collection, an aliquot batch of 10–30 oocytes was fixed and stained according to following procedure: cumulus cells were removed by hyaluronidase (Sevac) treatment (247 TRU/ml) for 10 min followed by 4 min of vortexing and repeated pipetting with a narrow-bone micropipette. The oocytes were mounted on microscope slides with Vaseline stripes, covered with a coverslip and fixed in ethanol–acetic acid 3:1 for 24 h. Staining was performed with 2% orcein in 50% aqueous acetic acid, 1% sodium citrate. Following careful substitution of dye with 40% acetic acid the samples were observed using phase-contrast microscopy (NU Zeiss-Jena).

Myelin basic protein and histone H1 kinase double assay

Activities of histone H1 and MBP kinases reflecting maturation-promoting factor (MPF) and MAP kinase activities, respectively, were measured in oocytes by their capacity to phosphorylate external substrates, namely histone H1 and MBP. At each time interval during the culture, 10 oocytes per sample were collected, washed (4×) in PBS and transferred in 3 µl of PBS into Eppendorf tubes. Samples were immediately frozen on dry ice and stored at –80 °C until assays were performed. The histone H1 and MBP kinase activities were measured according to Motlík *et al.* (1996). Briefly, 5 µl of buffer A (40 mM MOPS, pH 7.2, 20 mM para-nitrophenyl phosphate, 40 mM beta-glycerophosphate, 10 mM EGTA, 0.2 mM EDTA, 2 mM DTT, 0.2 mM Na_3VO_4, 2 mM benzamidine, 40 µg/ml leupeptin and 40 µg/ml aprotinin) were added to each sample and the samples subjected to three rounds of freezing and thawing on dry ice. After the final thawing the tubes were briefly vortexed and centrifuged at 10 000 *g* for 15 s. The kinase reaction was initiated by addition of 5 µl of buffer B (100 mM MOPS, pH 7.2, 20 mM para-nitrophenylphosphate, 40 mM beta-glycerophosphate, 20 mM $MgCl_2$, 10 mM EGTA, 0.2 mM EDTA, 5 µM cAMP dependent protein kinase inhibitor, 2 mM benzamidine, 40 µg/ml leupeptin and 40 µg/ml aprotinin; 600 µM ATP, 2 mg histone H1/ml and 3 mg MBP/ml) with 500 µCi/ml [γ-^{32}P] ATP (10 mCi/ml, Amersham). The reaction was conducted for 30 min at 30 °C and terminated by the addition of 10 µl 2× concentrated SDS PAGE sample buffer and boiling for 3 min. After electrophoresis on 15% SDS PAGE gel (Laemmli, 1970), the gels were stained with Coomassie Blue R250, destained overnight, dried and autoradiographed.

SDS PAGE and immunoblotting

Immunoblotting with antibodies against p44 (ERK1) and p42 (ERK2), two members of the MAP kinase family, was carried out to confirm that the activity of MBP kinase reflected that of MAP kinase. At each; time interval, 10 oocytes per sample were collected, washed five times in protein-free media (PBS), lysed in 10 µl 2× SDS sample buffer containing 5% 2-mercaptoethanol and stored immediately at –80 °C until electrophoresis. Samples were separated on 9% SDS PAGE gels (Laemmli *et al.*, 1970) in which the acrylamide:bis-acrylamide ratio in the separation gel was 100:1. Separated proteins were transblotted to Immobilon-P (Millipore) membranes using a tank-buffer apparatus (200 mA, 1 h). Blots were incubated in 10% teleost gelatin (Sigma) dissolved in 0.05% Tween-20 in Tris-buffered saline, pH 7.4 (TTBS) for 1 h prior to develop-

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ment with anti ERK-1 antibody (Santa Cruz, CA; sc-94, 1:1000), followed by secondary anti-rabbit, horseradish peroxidase-linked Ig (Amersham, 1:5000); the blots were incubated with each antibody for 1 h at room temperature. The blots were then washed at least 5 times for at least 10 min for each wash in TTBS and after that developed with an ECL chemiluminescent kit according to the manufacturer's instructions (Amersham).

Results

Activation of pig and cattle oocytes by BL I

The ability of BL I to activate pig and cattle oocytes was assessed on the basis of the oocytes' capability to develop to the pronuclear stage, or to a 2-cell stage embryo. The unaged MII oocytes (cultured 24 h for cattle and 46 h for pig in control medium) were treated with BL I for different time intervals (as indicated in figure legends) and then either fixed for morphological observations immediately, or subsequently cultured for additional time intervals in control medium after BL I treatment and then fixed. The data from four experiments for each species were pooled; the results are summarised in Tables 1 and 2 and in Figs. 1 and 2.

From the results shown, it seems clear that precise timing of the BL I treatment is essential for obtaining the best activation rates. Generally, intervals longer than 3 h were needed to induce activation of unaged MII oocytes. When the BL I treatment was prolonged beyond 5 h or 8 h, for cattle and pig oocytes respec-

Table 1 Morphology of cattle MII oocytes treated with butyrolactone I

	C24	C30	C42	BL3	BL5	BL8	BL10	BL12	BL16	BL5+C3	BL5+C5	BL5+C7	BL5+C11
Total no. of oocytes	27	20	28	37	34	41	32	50	38	40	38	37	47
MI	3	2	4		5		3	2			5		1
MII (MIII)	24	18	20	13	2	4	9	2	1	10	5	12	8
Ana II/Telo II				12	10	3				4		2	6
1 PN 1 PB					1			3		1	2	4	4
1 PN 2 PB			4	11	12	24	18	15	4	22	23	17	15
2 PN 1 PB				1	4	2		2		2	3	2	3
2 PN 2 PB								2	2				3
2-cell						1							1
Fragmented/Abnormal						7	2	24	31	1			6
Total no. of oocytes in pronuclear stage (%)			4 (14)	12 (32)	17 (50)	26 (66)	18 (56)	22 (44)	6 (16)	25 (63)	28 (74)	23 (62)	26 (55)

Oocytes were cultured *in vitro* for 24–42 h (C24–C42) and the 24 h cultured oocytes treated either with BL I alone for 3–16 h (BL3–BL16), or with BL for 5 h and subsequently cultured in control medium for 3–11 h (BL5+C3–BL5+C11).

Table 2 Morphology of pig MII oocytes treated with butyrolactone I

	C46	C70	BL3	BL5	BL8	BL24	BL8+C8	BL8+C10	BL8+C12	BL8+C16	BL10+C12
Total no. of oocytes	32	35	44	55	37	49	37	32	49	31	37
MI	3	2		2		1		3	2		
MII	27	31	2	10							
Ana II/Telo II	2		25	9		2				3	3
1 PN 1 PB						4	5	3	2		
1 PN 2 PB		2	17	28	23	25	16	9	11		
2 PN 1 PB				6	7	5	7	5	11		
2 PN 2 PB							2				
2 cell									6	16	9
Fragmented/Abnormal					7	12	7	12	17	12	25
Total no. of oocytes activated (%)			17 (39)	34 (62)	30 (81)	34 (69)	30 (81)	17 (53)	30 (61)	19 (51)	9 (24)

Oocytes were cultured *in vitro* for 46–70 h (C46–C70) and the 46 h cultured oocytes further treated either with BL I alone for 3–24 h (BL3–BL24) or with BL for 8 h and subsequently cultured in control medium for 8–16 h (BL8+C8–BL10+C12).

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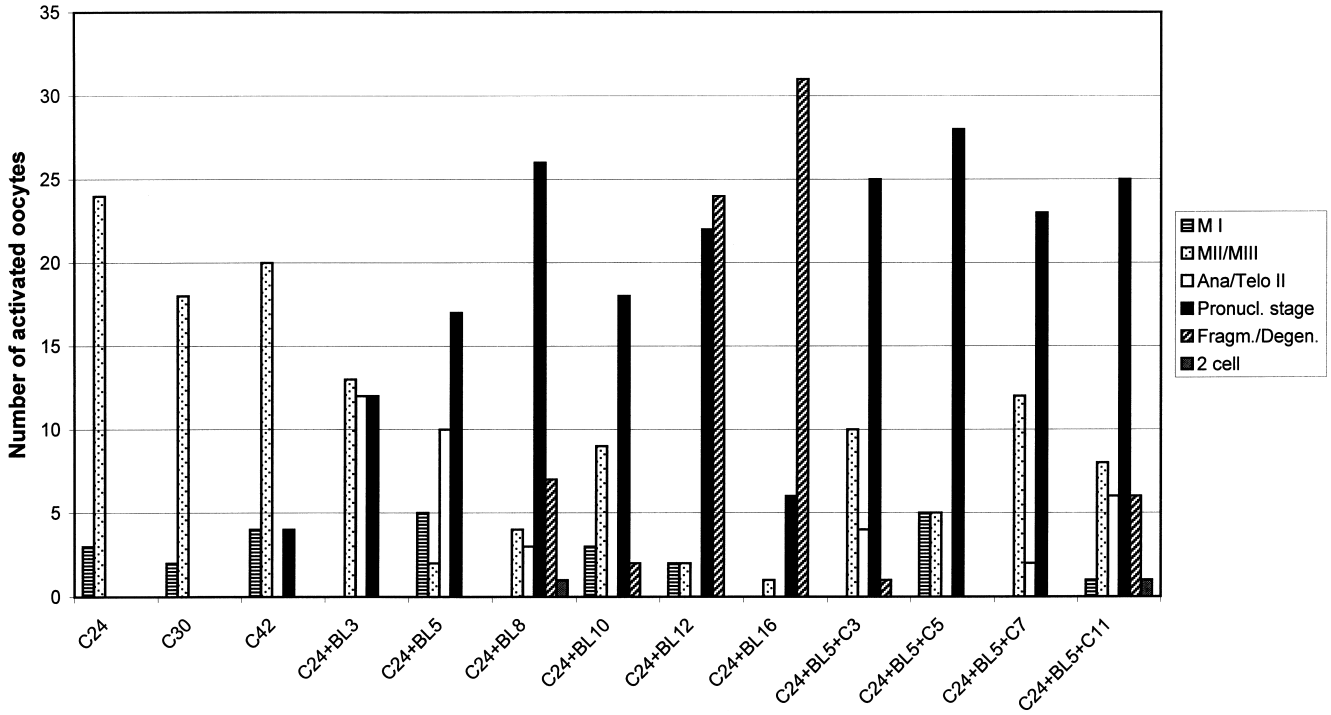

Figure 1 Morphology of cattle MII oocytes treated with BL I (data taken from Table 1). Oocytes were cultured *in vitro* for 24–42 h in control medium (C24–C42), and the 24 h cultured oocytes subjected to BL I treatment alone (C24+BL3–C24+BL16), or followed by subsequent culture in control medium (C24+BL5+C2–C24+BL5+C11).

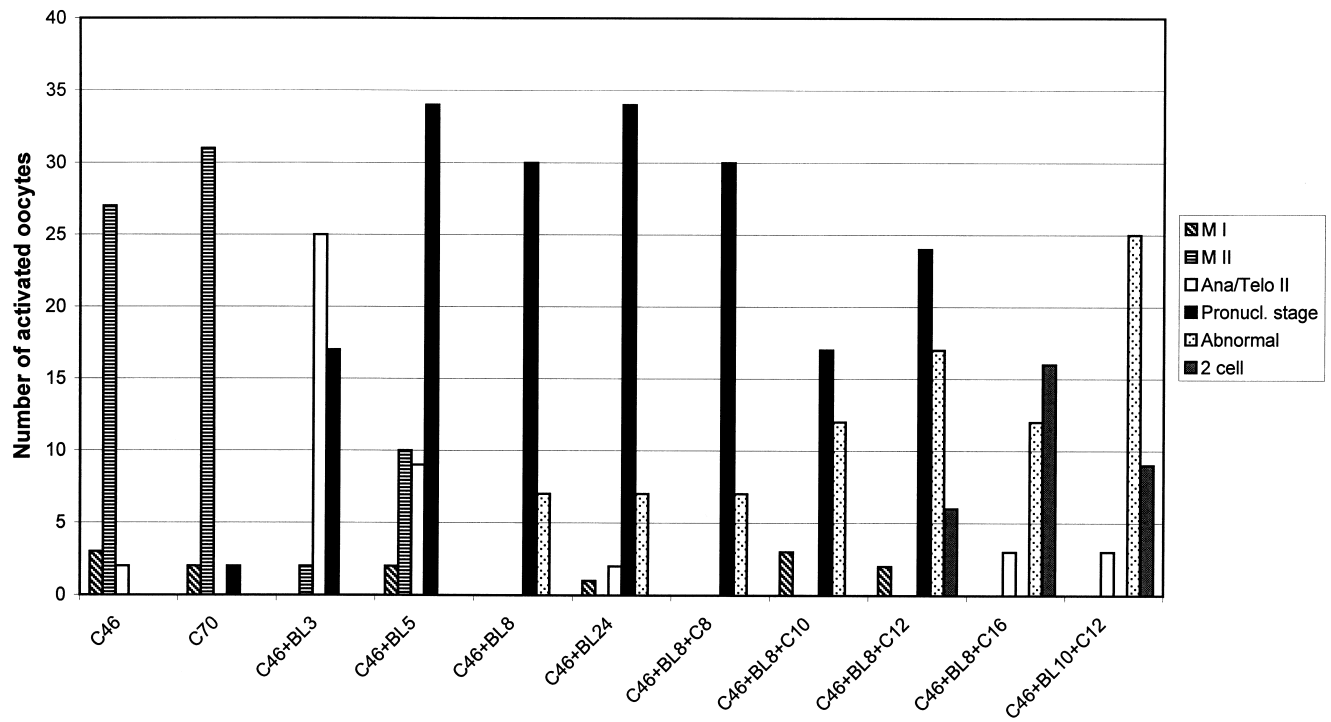

Figure 2 Morphology of pig MII oocytes treated with BL I (data taken from Table 2). Oocytes were cultured *in vitro* for 46–70 h in control medium (C46–C70), and the oocytes cultured for 46 h in control medium subjected to BLI treatment alone (C46+BL3–C46+BL24), or followed by subsequent culture in control medium (C46+BL8+C8–C46+BL10+C12).

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tively, the proportion of degenerated or abnormal oocytes increased proportionally. Optimum results were obtained when cattle MII oocytes were treated for 5 h with BL I and subsequently for 3–11 h in control medium, and pig MII oocytes for 8 h in BL I and then for 8–16 h in control medium; the percentage of activated oocytes after such treatment varied between 55% and 74% and between 53% and 81% for cattle and pig oocytes, respectively (Table 1, 2; Figs. 1, 2). However, in the case of cattle oocytes, even treatment with BL I for 5 h was not sufficient to activate a proportion of the oocytes (between 19% and 32%), and after the release of inhibitor and subsequent culture in control medium, such oocytes were found to be blocked in metaphase, i.e. some of them returned to MII stage and some extruded a second polar body and subsequently formed a MIII plate (data not shown; Table 1, Fig. 1).

Histone H1 and MBP kinase activities in pig and cattle oocytes treated with BL I

To assess the state of activation of two major protein kinases regulating the cell cycle in pig and cattle oocytes (cdc2 kinase and MAP kinase) after BL I treatment, the aliquot batch of 10 oocytes was used from each time interval for double kinase assay with histone H1 and MBP as external substrates.

The results reflected quite closely the morphological observations: as seen from Fig. 3*A*, both kinases were highly activated in cattle MII oocytes (some inactivation occurred in aged MII oocytes cultured for a prolonged 44 h period in total), after BL I treatment for 3 and 5 h both kinases were gradually inactivated (H1 kinase more quickly), and after 8 and 10 h in BL I no H1 kinase or MBP kinase activities were detected in such treated oocytes. Partial activation of both kinases in oocytes cultured in the control medium after removal from BL I block corresponds to the fact that a proportion of oocytes in these samples were blocked in metaphase (and as such possessed high H1 kinase and MBP kinase activities).

Similarly, both kinases were highly activated in pig MII oocytes (Fig. 4*A*), with partial inactivation after prolonged culture (up to 70 h). After BL I treatment both kinases were gradually inactivated; total inactivation of H1 kinase occurred already after 3 h, while MBP kinase remained partially active even after 5 h. The partial reoccurrence of H1 kinase activity after the removal of BL I block and culture in control medium (especially after longer intervals in control medium) is probably due to the presence of a small proportion of embryos which had already reached the first mitosis.

Western blot analysis of MAP kinase activity in pig and cattle oocytes treated with BL I

Since MBP is a substrate for a number of protein kinases, the activity of MAP kinase was also assayed by immunoblotting using an antibody recognising two members of the MAP kinase family: ERK1 and ERK2. Both these kinases are activated by phosphorylation, which results in a decrease in electrophoretic mobility, i.e. when the kinase becomes activated, a band shift to higher molecular weights could be seen on the blot. Figs. 3*B* and 4*B* show that the blots were in good agreement with results obtained in kinase assays: both cattle and pig MII-blocked oocytes possessed high levels of MAP kinase activities. BL I-mediated activation of oocytes resulted in gradual inactivation of MAP kinase, though much slower than that of histone H1 kinase. In both pig and cattle oocytes MAP kinase was still active after 3 h of BL I treatment and partial activity could be seen even after 5 h. After 8 h of BL I treatment the MAP kinase was totally inactivated (Figs. 3*B*, 4*B*). When cattle oocytes were treated for 5 h with BL I and then cultured in control medium for 3–15 h, partial activity of MAP kinase was detected in these samples, which is probably due to the above-mentioned fact that some oocytes from these batches were blocked at the MII/III stage (Fig. 3*B*). On the other hand, no activation was detected in pig oocytes cultured for 2–16 h in control medium after 8 h of BL I treatment (Fig. 4*B*), suggesting that MAP kinase activation does not occur during the first mitosis.

Discussion

In this study we show the activation of unaged (freshly matured) pig and cattle oocytes by a specific inhibitor of cdk kinases, BL I. We document that BL I is able to induce activation of both pig and cattle MII oocytes at high rates in a manner dependent on exposure time; however, precise timing of BL I treatment is required for best activation results: too short an exposure to BL I can lead to re-entry to the metaphase state (MII or MIII) while too long an exposure can lead to fragmentation or degeneration of oocytes. We also describe temporal changes in the activities of two major cell-cycle-related protein kinases – cdc2 kinase and MAP kinase – in these oocytes during activation by BL I. We show that the activation of oocytes (assessed on the basis of pronuclear formation) is accompanied by inactivation of both kinases, though inactivation of cdc2 kinase occurs more quickly than that of MAP kinase: while cdc2 kinase is inactivated after 3 h of BL I treatment, MAP kinase activity can be detected even after 5 h of treatment. This phenomenon is more pronounced in pig oocytes, in which cdc2 kinase is barely

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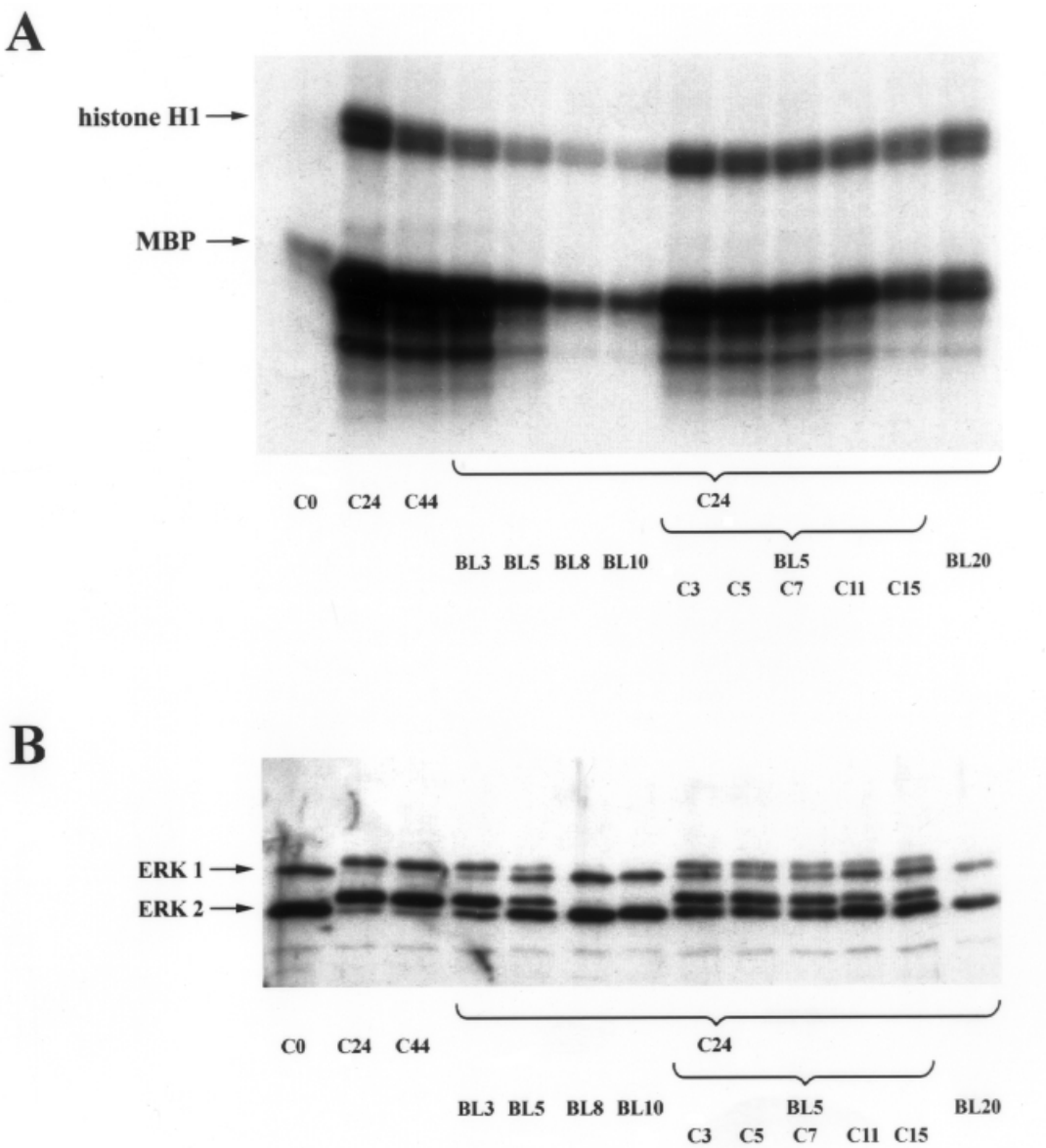

Figure 3 Histone H1 kinase and MAP kinase activities in control and BL I-treated oocytes. (*A*) Cattle oocytes were cultured in control medium for 0–44 h or in control medium for 24 h and then in BL I for 3–20 h, or 24 h in control medium and 5 h in BL I followed by transfer back to control medium for 3–15 h, and at the indicated time intervals the samples were collected for histone H1 and MBP double kinase assay by adding both substrates in a kinase cocktail. (*B*) Changes in phosphorylation status of two MAPKs (ERK1 and ERK2) as detected by mobility shifts in samples from control and BL I-treated oocytes shown by western blotting. Ten oocytes were used for each sample in both assays. The experiment was conducted three times and a representative example is shown.

detectable after 3 h, although MAP kinase is still fully active after 5 h. It should be noted that BL I acts on the ATP binding site and thus inhibits the instantaneous activity of the cdc2 kinase (and other cdk kinases except cdk4 and cdk7) *in situ*, but does not directly influence the activation state of the kinase (Kitagawa *et al.*, 1993). This explains the fact that we were able to observe some cdc2 kinase activity in the samples prepared from cattle oocytes treated for 3 h with BL I.

The mechanisms by which oocytes are activated are not fully understood, though it is generally believed that the key event in this process is inactivation of CSF by increased intracellular Ca^{2+} and subsequent inactivation of MPF (cdc2 kinase) via cyclin degradation. As mentioned earlier, several papers have reported activation of unaged pig or cattle oocytes by a combination of different stimuli, such as ionomycin/ionophore, ethanol or electric pulse combined with 6-DMAP or cycloheximide (Presicce & Yang, 1994; Tanaka & Kana-

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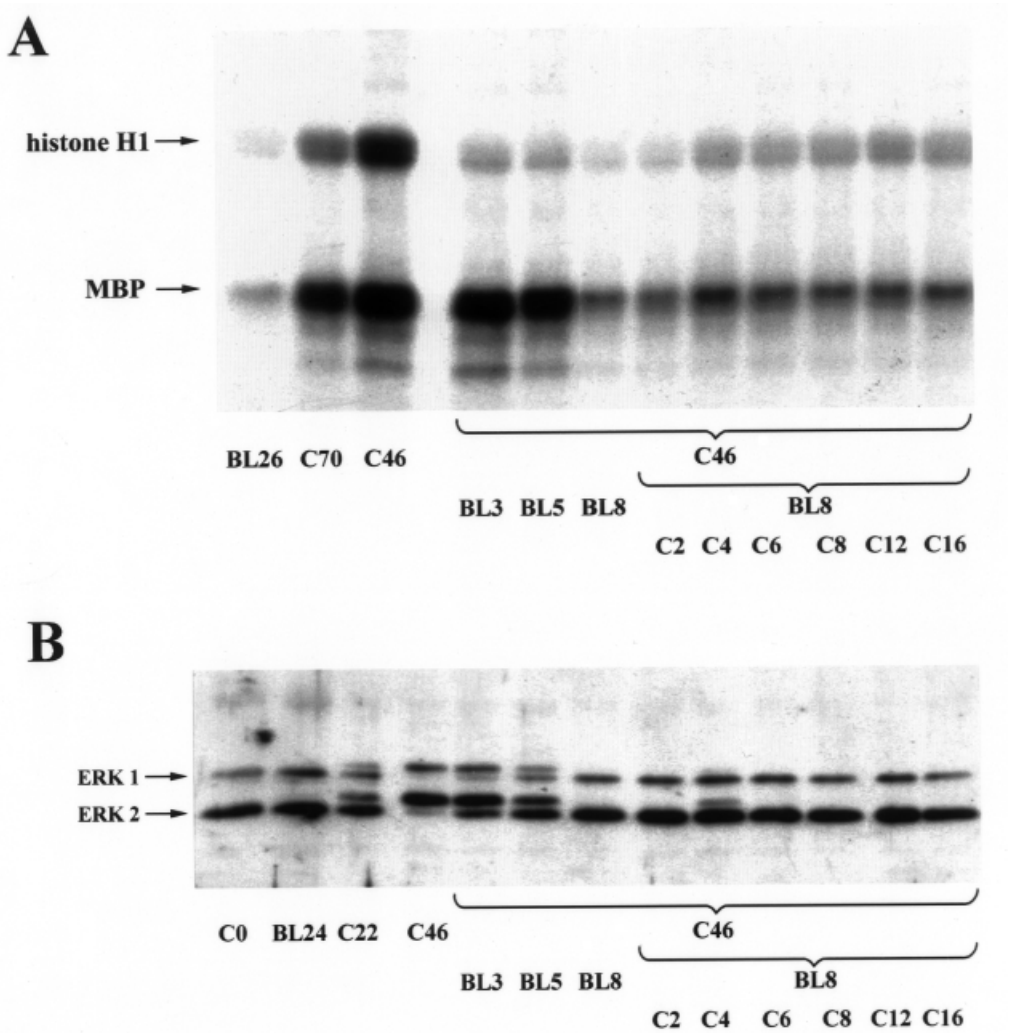

Figure 4 Histone H1 kinase and MAP kinase activities in control and BL I-treated oocytes. (*A*) Pig oocytes were cultured in control medium for 0–70 h or in control medium for 46 h and then in BL I for 3–8 h, or 46 h in control medium, then 8 h in BL I followed by transfer back to control medium for 8–16 h, and at the indicated time intervals the samples were collected for histone H1 and MBP double kinase assay by adding both substrates in a kinase cocktail. (*B*) Changes in phosphorylation status of two MAPKs (ERK1 and ERK2) as detected by mobility shifts in samples from control and BL I-treated oocytes shown by western blotting. Ten oocytes were used for each sample in both assays. The experiment was conducted three times and a representative example is shown.

gawa, 1997; Susko-Parrish *et al*., 1994; Cha *et al*., 1997; Liu *et al*., 1998; Leal & Liu, 1998; Ernst *et al*., 1999). These treatments were used with the intention of influencing both Ca^{2+} metabolism and cyclin B turnover. Both cycloheximide and 6-DMAP, however, apart from influencing cdc2/cyclin B kinase, nonspecifically influence a number of other proteins and protein kinase activities, which might impair further developmental capacity of resulting parthenotes. For that reason, more recently, specific inhibitors of cdk kinases (bohemine and BL I) have been used in combination with ionomycin (Alberio *et al*., 2000) or with electric pulse (Dinnyes *et al*., 2000) for activation of cattle and pig oocytes, respectively. While bohemine treatment alone had only small potential for activating bovine MII oocytes (Alberio *et al*., 2000), BL I at a concentration of 150–200 μM was shown to be able to activate pig oocytes at quite high rates (Dinnyes *et al*., 2000). Dinnyes *et al*. (2000) also showed that the combination of BL I with an electric pulse did not significantly increase the rates of activation; however, the nuclear configuration was significantly changed: while activation by BL I alone resulted in 13–20% of parthenotes forming a haploid pronucleus and two polar bodies and 47–53% of parthenotes forming two pronuclei without extrusion of the second polar body, the combination of BL I with an electric pulse induced the formation of two pronuclei and one polar body in the great majority of the parthenotes obtained. On the other hand, when bohemine was used alone for activation of bovine MII

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oocytes no chromosome segregation was observed and nearly all activated oocytes formed one diploid pronucleus without extrusion of the second polar body. The combination of bohemine and ionomycin induced the extrusion of the second polar body and the great majority of parthenotes contained one pronucleus and two polar bodies (Alberio *et al*., 2000).

In this paper we show that BL I alone at a concentration of 100 µM is able to induce activation of both pig and cattle MII oocytes at rates comparable to the above-mentioned reports. The majority of parthenotes (60–100% of cattle and 46–100% of pig pronuclear (PN)-stage parthenotes; see Tables 1, 2) formed one pronucleus and two polar bodies, 8–24% of cattle and 15–46% of pig PN-stage parthenotes formed two pronuclei and one polar body, suggesting that the segregation of chromosomes occurred in the vast majority of activated oocytes. The differences between our results and those of Dinnyes *et al.* (2000) are likely to be due to the different timing of exposure of the oocytes to BL I. From the presented data it is clear that the activation rates increase with prolonged exposure of the oocytes to BL I; however, after reaching certain threshold (8 h for cattle, 10 h for pig oocytes), the numbers of degenerated or abnormal oocytes significantly increase. On the other hand, too short an exposure to BL I at a concentration of 100 µM is not sufficient for inducing activation and the oocytes remain arrested in metaphase. We have therefore chosen 5 h and 8 h intervals for cattle and pig oocytes, respectively, to achieve optimal results. Apart from the timing arrangements, the differences between our results and those of Dinnyes *et al.* (2000) could also be caused by different doses of BL I. These authors speculate that the increased concentrations of BL I might affect the cytoskeleton, probably via direct non-specific inhibition of MAP kinase by BL I. Similarly, an electric pulse combined with BL I (Dinnyes *et al*., 2000) induces inactivation of CSF and as such also MAP kinase inhibition quite soon during the process of activation. Our results on the time course of cdc2 and MAP kinase activities during activation of pig and cattle oocytes by BL I indicate the following: firstly that cdc2 kinase becomes inactivated after 3 h of BL I exposure (in both pig and cattle oocytes), probably as a result of the absence of cdc/cyclin B activity *in situ*; secondly that MAP kinase is still active after 5 h of BL I exposure and becomes totally inactivated subsequent to cdc2 kinase – after about 5 h and 8 h of BL I exposure in cattle and pig oocytes, respectively. These results suggest that MAP kinase inactivation occurs as a consequence of cdc2/cyclin B inactivation and when compared with the combined activation protocol, it is somehow postponed. Moreover, the fact that MAP kinase, on the other hand, can impair the inhibitory control of myt1 kinase on cdc2 kinase activity via its substrate p90rsk (Palmer *et al*., 1998), explains our findings showing that in the oocytes treated for a shorter time (3 h) with BL I and then cultured in control medium, cdc2 kinase becomes reactivated and the oocytes return to the metaphase state. This could be due to the continued presence of high MAP kinase activity in such oocytes. When MAP kinase becomes inactivated after longer treatment with BL I, the inhibitory action of myt1 kinase is possibly re-established, cdc2 kinase remains at low levels and the oocytes become irreversibly activated.

Our results also confirm the generally known facts that the activation of unaged cattle oocytes is more difficult in comparison with pig oocytes and that the activation rates for cattle oocytes are lower than those for pig oocytes, even when the concentration and timing of BL I exposure are optimised. The greater suitability of BL I alone or in combination with other activation stimuli for activation and/or cloning experiments needs to be determined for both species in further developmental studies.

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References

Abrieu, A., Lorca, T., Labbe, J.C., Morin, N., Keyse, S. & Doree, M. (1996). MAP kinase does not inactivate, but rather prevents the cyclin degradation pathway from being turned on in *Xenopus* egg extracts. *J. Cell Sci.* **109**, 239–46.

Abrieu, A., Doree, M. & Picard, A. (1997). Mitogen-activated protein kinase activation down-regulates a mechanism that inactivates cyclin B-cdc2 kinase in G_2-arrested oocytes. *Mol. Biol. Cell* **8**, 249–61.

Alberio, R., Kulbelka, M., Zakhartchenko, V., Hajdúch, M., Wolf, E. & Motlík, J. (2000). Activation of bovine oocytes by specific inhibition of cyclin dependent kinases. *Mol. Reprod. Dev.* **55**, 422–43.

Cha, S.K., Kim, N.H., Lee, S.M., Baik, C.S., Lee, H.T. & Chung, K.S. (1997) Effect of cytochalasin B and cycloheximide on the activation rate, chromosome constituent and *in vitro* development of porcine oocytes following parthenogenetic stimulation. *Reprod. Fertil. Dev.* **9**, 441–6.

Ciemerych, M.A., Maro, B. & Kubuak, J.Z. (1999). Control of duration of the first two mitoses in a mouse embryo. *Zygote*, **7**, 293–300.

Clarke, H.J. & Masui, Y. (1983). The induction of reversible chromosome decondensation by protein synthesis inhibition during meiotic maturation of mouse oocytes. *Dev. Biol.* **97**, 291–301.

Collas, P., Sullivan, E.J. & Barnes, F.L. (1993). Histone H1 kinase activity in bovine oocytes following calcium stimulation. *Mol. Reprod. Dev.* **34**, 224–31.

Collas, P., Chang, T., Long, C. & Robl, J.M. (1995). Inactivation of histone H1 kinase by Ca^{2+} in rabbit oocytes. *Mol. Reprod. Dev.* **40**, 253–8.

Cuthberson, K.S. (1983). Parthenogenetic activation of mouse oocytes *in vitro* with ethanol and benzyl alcohol. *J. Exp. Zool.* **226**, 311–14.

Dinnyes, A., Hirao, Y. & Nagai, T. (2000). Parthenogenetic activation of porcine oocytes by electric pulse and/or butyrolactone I treatment. *Cloning* **1**, 209–16.

Ernst, C.A., Liebfried-Rutledge, L.M. & Dentine, M.R. (1999). Development of an efficient method to produce uniformly haploid parthenogenones. *J. Exp. Zool.* **284**, 112–18.

Fukla, J. Jr., Liebfried-Rutledge, L.M. & First, N.L. (1991). Effect of 6-dimethylaminopurine on germinal vesicle breakdown of bovine oocytes. *Mol. Reprod. Dev.* **29**, 379–84.

Haccard, O., Sarcevic, B., Lewellyn, A., Hartley, R., Roy, L., Izumi, T., Erikson, E. & Maller, J.L. (1993). Induction of metaphase arrest in cleaving *Xenopus* embryos by MAP kinase. *Science* **262**, 1262–5.

Hagen, D.R., Prather, R.S. & First, N.L. (1991). Response of porcine oocytes to electrical and chemical activation during maturation *in vitro*. *Mol. Reprod. Dev.* **28**, 70–3.

Homa, S.T., Caroll, J. & Swann, K. (1993). The role of calcium in mammalian oocyte maturation and egg activation. *Hum. Reprod.* **8**, 1274–81.

Kikuchi, K., Izaike, Y., Noguchi, J., Furukawa, T., Daen, F.P., Naito, K. & Toyoda, Y. (1995). Decrease of histone H1 kinase activity in relation to parthenogenetic activation of pig follicular oocytes matured and aged *in vitro*. *J. Reprod. Fertil.* **105**, 325–30.

Kitagawa, M., Okabe, T., Ogino, H., Matsumoto, H., Suzuki-Takahashi, I., Kokubo, T., Higashi, H., Saitoh, S., Taya, Y., Yasuda, H., Ohba, Y., Nishimura, S., Tanaka, N. & Okuyama, A. (1993). Butyrolactone I, a selective inhibitor of cdk2 and cdc2 kinase. *Oncogene* **8**, 2425–32.

Kline, D. & Kline, J.T. (1992). Repetitive calcium transients and the role of calcium in exocytosis and cell cycle activation in the mouse egg. *Dev. Biol.* **149**, 80–9.

Kono, T., Jones, K.T., Bos-Mikich, A., Whittingham, D.G. & Carroll, J. (1996). A cell cycle-associated change in Ca^{2+} releasing activity leads to the generation of Ca^{2+} transients in mouse embryos during the first mitotic division. *J. Cell Biol.* **132**, 915–23.

Kubelka, M., Motlík, J., Schultz, R.M. & Pavlok, A. (2000). Butyrolactone I reversibly inhibits meiotic maturation of bovine oocytes, without influencing chromosome condensation activity. *Biol. Reprod.* **62**, 292–302.

Kubiak, J.Z. (1989). Mouse oocytes gradually develop the capacity for activation during the metaphase II arrest. *Dev. Biol.* **136**, 537–45.

Laemmli, U.K. (1970). Cleavage of structure proteins during the assembly of the head of bacteriophage T4. *Nature* **227**, 680–5.

Leal, C.L. & Liu, L. (1998). Differential effects of kinase inhibitor and electrical stimulus on activation and histone H1 kinase activity in pig oocytes. *Anim. Reprod. Sci.* **52**, 51–61.

Liu, L., Ju, J.C. & Yang, X. (1998). Differential inactivation of maturation-promoting factor and mitogen-activated protein kinase following parthenogenetic activation of bovine oocytes. *Biol. Reprod.* **59**, 537–45.

Lorca, T., Cruzalegui, F.H., Fesquet, D., Cavadore, J.C., Mery, J., Means, A. & Doree, M. (1993). Calmodulin-dependent protein kinase II mediates inactivation of MPF and CSF upon fertilization of *Xenopus* eggs. *Nature* **366**, 270–3.

Moos, J., Visconti, P.E., Moore, G.D., Schultz, R.M. & Kopf, G.S. (1995). Potential role of mitogen-activated protein kinase in pronuclear envelope assembly and disassembly following fertilization of mouse eggs. *Biol. Reprod.* **53**, 692–9.

Moos, J., Kopf, G.S. & Schultz, R.M. (1996). Cycloheximide-induced activation of mouse eggs: effects on cdc2/cyclin B and MAP kinase activities. *J. Cell Sci.* **109**, 739–48.

Motlík, J., Šutovskeý, P., Kalous, J., Kubelka, M., Moos, J. & Schultz, R.M. (1996). Co-culture with pig membrana granulosa cells modulates the activity of cdc2 and MAP kinase in maturing cattle oocytes. *Zygote* **4**, 247–56.

Nagai, T. (1987). Parthenogenetic activation of cattle follicular oocytes *in vitro* with ethanol. *Gamete Res.* **16**, 243–9.

Nurse, P. (1990). Universal control mechanism regulating onset of M-phase. *Nature* **344**, 503–8.

Palmer, A., Gavin, A.C. & Nebreda, A.R. (1998). A link between MAP kinase and p34(cdc2)/cyclin B during oocyte maturation: p90(rsk) phosphorylates and inactivates the p34(cdc2) inhibitory kinase Myt1. *EMBO J.* **17**, 5037–47.

Pavlok, A., Lucas-Hahn, A. & Niemann, H. (1992). Fertilization and developmental competence of bovine oocytes derived from different categories of antral follicles. *Mol. Reprod. Dev.* **31**, 63–7.

Pavlok, A., Kaláb, P. & Bobák, P. (1997). Fertilization competence of bovine normally matured or aged oocytes derived from different antral follicles: morphology, protein synthesis, H1 and MBP kinase activity. *Zygote* **5**, 235–46.

Pines, J. & Hunter, T. (1990). $p34^{cdc2}$: the S and M kinase? *New Biol.* **2**, 389–401.

Presicce, G.A. & Yang, X. (1994). Nuclear dynamics of parthogenesis of bovine oocytes matured *in vitro* for 20 and 40 hours and activated with combined ethanol and cycloheximide treatment. *Mol. Reprod. Dev.* **37**, 61–8.

Siracusa, G., Whittingham, D.G., Molinaro, M. & Vivarelli, E. (1978). Parthenogenetic activation of mouse oocytes induced by inhibitors of protein synthesis. *J. Embryol. Exp. Morphol.* **43**, 157–66.

Susko-Parrish, J.L., Liebfried-Rutledge, M.L., Northey, D.L., Schutzkus, V., & First, N.L. (1994). Inhibition of protein kinases after an induced calcium transient causes transition of bovine oocytes to embryonic cycles without meiotic completion. *Dev. Biol.* **166**, 729–39.

Steinhardt, R.A., Epel, D., Carroll, E.J. Jr. & Yanagimachi, R. (1974). Is calcium ionophore a universal activator for unfertilized eggs? *Nature* **252**, 41–3.

Tanaka, H. & Kanagawa, H. (1997). Influence of combined

activation treatments on the success of bovine nuclear transfer using young or aged oocytes? *Anim. Reprod. Sci.* **49**, 113–23.

Tateno, H. & Kamiguchi, Y. (1997). Parthenogenetic activation of Chinese hamster oocytes by chemical stimuli and its cytogenetic evaluation. *Mol. Reprod. Dev.* **47**, 72–8.

Thibier, C., De Smedt, V., Poulhe, R., Huchon, D., Jessus, C. & Ozon, R. (1997). *In vivo* regulation of cytostatic activity in *Xenopus* metaphase II-arrested oocytes. *Dev. Biol.* **185**, 55–66.

Verlhac, M.H., Kubiak, J.Z., Clarke, H.J. & Maro, B. (1994). Microtubule and chromatin behavior follow MAP kinase activity but not MPF activity during meiosis in mouse oocytes. *Development* **120**, 1017–25.

Verlhac, M.H., Kubiak, J.Z., Weber, M., Geraud, G., Colledge, W.H., Evans, M.J. & Maro, B. (1996). Mos is required for MAP kinase activation and is involved in microtubule organization during meiotic maturation in the mouse. *Development* **122**, 815–22.

Ware, C.B., Barnes, F.L., Maiki-Laurila, M. & First, N.L. (1989). Age dependence of bovine oocyte activation. *Gamete Res.* **22**, 265–75.

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